REMARKS

Claims 3, 4, 8, 9, 11, 19-23, 25-26, 38-43, 45-57, 59-62, 74-78 and 101-110 remain in this case.

Claims 3, 4, 8, 9, 11, 19-23, 25-27, 38-42, 74-78, 101-105 and 107 have been rejected under 35 USC 112, second paragraph.

The claims under consideration, with the exception of claims 108-110, have been rejected as either anticipated by Razavi '685, obvious over Razavi '685 in view of McNamara '370, obvious over Razavi '685 in view of Hanson '352, or obvious over Zukowski '755 in view of Kropf '849 and either Ragheb '904 or Hanson '352.

Claim 27 has been canceled as a duplicate of claim 39.

Claims 38, 43 and 74 have been amended to specify that the claimed prosthesis is for use within a blood vessel and the claimed methods are carried out inside a blood vessel.

Claims 105-107 have been amended for consistency in nomenclature.

New claims 109 and 110, which correspond to claims 105 and 107 but depend from claims 101 and 103, respectively, have been added to emphasize that a NO generator may be contained within open spaces defined within the sleeve interior.

The Cited Art

Razavi U.S. Patent No. 5,676,685 discloses a removable, temporary stent 10 comprising a wire coil 12 enclosed within a biodegradable/bioabsorable coating 14. Coating 14 includes outer and inner layers 16, 18. Outer layer 16 may include various agents (column 3, lines 22-30). Inner layer 18 surrounds a wire coil 12 and is made of a material that can be softened or liquefied when heated to permit wire coil 12 to be pulled out from coating 14 leaving coating 14 in place. "Removal of core wire 12 will of course be accomplished at such time as the stent has served its temporary purpose." Column 2, lines 34-36.

Zukowski International Application Publication WO 97/40755 is directed to a device used to repair defective venous valves. The device is positioned externally around the vein at the site of the defective valve. The device provides an external, constricting force to the vein to partially flatten the vein to an oval shape to help restore proper valve operation. Figures 15-18 show a helical support which "is deformed by compression to assume the transversely elliptical cross-section." Page 9, lines 32-33. Accordingly, the Zukowski publication does not disclose an endoluminal prosthesis because it is used outside, not inside a lumen.

Kropf U.S. Patent No. 4,760,849 discloses a planar blank which can be made into a coil spring useful as a filter for thromboses. The coil spring has apertures to facilitate ingrowth of tissue into the spring material. See column 1, lines 61-63 and column 2, lines 51-55. This reference only discloses a stent. It teaches away from adding a graft material because a stated intention of the invention is to permit tissue ingrowth through the apertures. There is no recognition that the addition of a graft material would be useful or possible.

Ragheb U.S. Patent No. 5,873,904 discloses a medical device 10 including a structure 12, typically a vascular stent 12, composed of an elastic/non-elastic, biodegradable/non-biodegradable base material 14, such as stainless steel, nitinol, polymers, etc. Stent 12 is shown to have several layers of materials coated thereon. At least one layer 18 of a bioactive material is on the surface of stent 12. An outer porous layer 20 is on layer 18 to provide controlled release of the bioactive material. A porous/non-porous layer 16 may be used between the bioactive layer 18 and stent 12. A second bioactive layer 22 may be used between porous layer 20 and bioactive layer 18; if so, an inner porous layer 24 may be used between the bioactive layers 18, 22.

McNamara U.S. Patent No. 5,147,370 is cited as disclosing a coil with turns touching one another. However, McNamara discloses a solid metal, specifically nitinol, coil stent having a rectangular cross-section to create tight junctions between the coils "which decreases or prevents ingrowth of invasive cancer tissue through the implanted device or deposition of undesirable blood components on underlying tissue damage to by angioplasty or other procedures used to clear clogged blood vessels." See column 3, lines 40-41 and column 5, lines 21-30. Therefore, it would have been contrary to the teachings of McNamara to create a stent that encourages tissue ingrowth.

Hanson U.S. Patent No. 5,399,352 discloses delivering a drug, such as nitric oxide liberators, into a blood vessel by replacing a portion of the blood vessel with a porous vascular graft 4 (figures 1-4). A reservoir 20 of the drug is created between a porous portion 28 of vascular graft 4 and a second element 24. In a second embodiment a vascular patch 104 (figures 5-7) is placed over an aperture 107 formed in an artery. A reservoir 120 of the drug is created between a porous portion 128 of patch 104 and a second element 124.

The Rejections Responded To

1. Claim Rejections-35 USC §112

Claims 3, 4, 8, 9, 11, 19-23, 25-27, 38-42, 74-78, 101-105 and 107 have been rejected under 35 USC 112, second paragraph, as being indefinite.

Applicant does not agree that the use of "at least one of" makes claim 38 and 74 ambiguous. The inner surface is a surface, while the sleeve interior is a region. However, to expedite prosecution of this case and allowance to an issued patent, claims 38 and 74 have been amended to recite that the biologically active agent is within the sleeve interior, and that the agent passes from the interior, through the material and into the blood vessel. It is believed that these amendments should remove the Examiner's basis for concern.

2. Claim Rejections-35 USC §102

Claims 3, 9, 26, 27, 38-41, 74-77, 101 and 102 were rejected under 35 USC 102 (b) as being anticipated by Razavi '685.

Independent apparatus claim 38. First, Examiner has taken the position that Razavi '685 discloses an anti-thrombotic drug associated with material 14. However, Razavi '685 teaches that material 14 "may also include quantities of such materials as: anti-thrombotic, anti-platelet, ..." Column 3, lines 22-23. That is, Razavi '685 teaches the incorporation of a drug into the composition of material 14. There is nothing in Razavi '685 disclosing or suggesting the use of:

> "a dispensable, biologically active agent within the sleeve interior, said dispensable agent being dispensable from the sleeve interior, through the inner surface, through the material, out of the outer surface and into a blood vessel of a patient."

Rather, in Razavi '685 material 14, used to coat coil 12, may include a biological agent incorporated therein. However, this is not what is claimed. That is, Razavi '685 does not disclose a biologically active agent within a sleeve interior (rather, it is incorporated into material 14) nor does it disclose that the biologically active agent be dispensable from this sleeve interior (there is no suggestion in Razavi '685 to place the agent within a sleeve interior), through the inner surface (the agent of Razavi '685 is incorporated into material 14 and therefore would not pass through the inner surface of material 14), through the material and out of the outer surface and into a blood vessel. There is no suggestion or reason to modify the device shown in Razavi '685 to arrive at this presently claimed structure. **Second,** the Examiner is taking the position that material 14 forms a coiled sleeve. Applicant disagrees that material 14 could be characterized as a sleeve as that term is commonly understood. "1. The part of a garment that covers all or part of the arm. 2. Any encasement or shell into which a piece of equipment fits." *The American Heritage Dictionary of the English Language, New College Edition*, Houghton Mifflin Company, 1976. Material 14 may be characterized as coating or layer, but not a sleeve. Accordingly, claim 38 is allowable over Razavi '685.

Dependent **apparatus claims 101, 102**. Claims **101 and 102** (parent: claim 38) more specifically define the open aspects of the sleeve interior to further distinguish the invention over the cited art. See figure 3A and page 11, line 30-page 12, line 2. There is nothing in Razavi '685 suggesting that the prosthesis include a sleeve interior comprising "regions occupied by the coiled body and open spaces not occupied by the coiled body" (claim 101) nor a sleeve interior which "is oversized relative to the coiled body so to loosely contain the coiled body" (claim 102). Accordingly, claims 101 and 102 are allowable over the cited art for these additional reasons.

Independent method claim 74. First, Examiner has taken the position that Razavi '685 discloses an anti-thrombotic drug associated with material 14. However, Razavi '685 teaches that material 14 "may also include quantities of such materials as: anti-thrombotic, anti-platelet, …." Column 3, lines 22-23. That is, Razavi '685 teaches the incorporation of a drug into the composition of material 14. There is nothing in Razavi '685 disclosing or suggesting the use of:

> delivering a coiled prosthesis to a target site inside a blood vessel of a patient, … the prosthesis comprising a coiled body …, a coiled sleeve of material …, and a dispensable, biologically active agent within the sleeve interior;…
>
> releasing the agent into the blood vessel, the agent passing from the interior, through the material and into the blood vessel.

Rather, in Razavi '685 material 14 is used to coat coil 12 and may include a biological agent incorporated therein. However, this is not what is claimed. That is, Razavi '685 does not disclose or suggest delivering a coiled prosthesis comprising a biologically active agent within a sleeve interior (rather, it is incorporated into material 14). Nor does Razavi '685 disclose releasing the biologically active agent from this sleeve interior (there is no suggestion in Razavi '685 to place the agent within a sleeve interior), through the material and into the blood vessel. Therefore, Razavi '685 does not suggest the coiled prosthesis delivering step with the recited placement of the agent nor the agent releasing step with "the agent passing from the interior, through the material and into the blood vessel." **Second,** the Examiner is taking the position that material 14 forms a coiled sleeve. Applicant disagrees that material 14 could be characterized as a sleeve as that term is commonly understood. "1. The part of a garment that covers all or part of the arm. 2. Any encasement or shell into which a piece of equipment fits." *The Amerian Heritage Dictionary of the English Language, New College Edition,* Houghton Mifflin Company, 1976. Material 14 may be characterized as coating or layer, but not a sleeve. Accordingly, claim 74 is also allowable over Razavi '685.

3. Claim Rejections-35 USC §103

Claims 4, 8, 19-23, 25, 38, 42, 43, 45, 47-57, 59-62, 74, 78, 103 and 104 were rejected under 35 USC 103 (a) as being unpatentable over Zukowski '755 in view of Kropf '849 and Ragheb '904.

Independent apparatus claim 38. First, Zukowski '755 is directed to a device used to repair defective venous valves. The Zukowski '755 device is positioned externally around the vein at the site of the defective valve. The Zukowski '755 device provides an external, constricting force to the vein to partially flatten the vein to an oval shape to help restore proper valve operation. Accordingly, one of ordinary skill in the art would not look to Zukowski '755 when faced with designing a device for use in a blood vessel. **Second**, there is nothing in the cited art disclosing or suggesting the use of:

> "a dispensable, biologically active agent within the sleeve interior, said dispensable agent being dispensable from the sleeve interior, through the inner surface, through the material, out of the outer surface and into a blood vessel of a patient."

Any agent dispensable from the device of Zukowski '755 would necessarily be dispensed from the material, through the inner surface of the device and to the outer surface of a blood vessel, substantially the opposite of what is claimed. Therefore, claim 38 is allowable over the cited art.

Dependent **apparatus claim 8**. Claim 8 (parent: claim 38) recites that the body has side members and connecting cross members. Kropf '849 discloses a solid metal stent having apertures to permit tissue ingrowth through the apertures. Therefore, Kropf '849 teaches away from the suggested combination of Kropf '849 and Zukowski '755 because covering of the stent of Kropf '849 with the material of Zukowski '755 could hinder or prevent the desired tissue ingrowth. In addition, there is nothing in Zukowski '755 suggesting that the additional structural characteristics provided by the ladder-type stent of Kropf '849 would have been useful or desirable. Accordingly, claim 8 is allowable over the cited art.

Dependent **apparatus claim 22**. Claim 22 (parent: claim 38) recites that at least half of the first agent is dispensable prior to the start of dispensing of the second agent; Ragheb '904 separates bioactive layers 18, 22 with a porous layer 24 so that the agent in bioactive layer 18 will start being dispensed when any portion of porous layer 24 is exposed. Therefore, Ragheb '904, as well as the other art a record, fails to teach or suggest the timing of the dispensing of the agents as specified in claim 22 so that claim 22 is allowable.

Dependent **apparatus claim 25**. Claim 25 (parent: claim 38) recites that the porous material has an inner surface that is substantially impervious to the passage of blood therethrough; this aspect is absent from the cited art. There is no factual support for the Examiner's position that the inner surface of the porous material of Zukowski '755 is inherently impervious to blood. In addition, because the

device of Zukowski '755 is used outside a blood vessel to aid the functioning of a malfunctioning venous valve within the blood vessel, there would have been no apparent reason to make the inner surface of the device of Zukowski '755 impervious to the flow of blood. Therefore, claim 25 is allowable over the cited art.

Independent method claim 43. First, as mentioned above, Zukowski '755 is directed to a device used to repair defective venous valves. The device is positioned externally around the vein at the site of the defective valve. The device provides an external, constricting force to the vein to partially flatten the vein to an oval shape to help restore proper valve operation. Accordingly, one of ordinary skill in the art would not look to Zukowski '755 when faced with designing a method for delivering a biologically active agent to a target site inside a blood vessel. **Second,** claim 43 recites delivering the coiled prosthesis to target site inside a blood vessel (as opposed to structure of Zukowski '755 being positioned on the outside a vein) and radially expanding the prosthesis to press against the wall of the blood vessel (as opposed to radially contracting the device of Zukowski '755). **Third**, there is nothing in the art suggesting "selecting a coiled prosthesis comprising a coiled body having longitudinally extending side members and cross members connecting said side members, ... ". Kropf '849 discloses a solid metal stent having apertures to permit tissue ingrowth through the apertures. Therefore, Kropf '849 teaches away from using "a material extending along a coiled path along the entire coiled body ..." because doing so could hinder or prevent the desired tissue ingrowth. In addition, there is nothing in Zukowski '755 suggesting that the additional structural characteristics provided by the ladder-type stent of Kropf '849 would have been useful or desirable. Therefore, the art lacks the appropriate teaching or suggestion to substitute the metal stent of Kropf '849 for the coiled wires of Zukowski '755. Therefore, claim 43 is allowable over the cited art.

Dependent **method claim 54**. Claim 54 (parent: claim 43) recites that the selecting step is carried out by selecting a prosthesis with the porous material having an inner surface that is substantially impervious to the passage of blood therethrough; this aspect is absent from the cited art. There is no factual support for the Examiner's position that the inner surface of the porous material of Zukowski '755 is inherently impervious to blood. In addition, because the device of Zukowski '755 is used outside a blood vessel to aid the functioning of a malfunctioning venous valve within the blood vessel, there would have been no apparent reason to make the inner surface of the device of Zukowski '755 impervious to the flow of blood. Therefore, claim 54 is allowable over the cited art.

Independent method claim 74. First, as mentioned above, Zukowski '755 is directed to a device used to repair defective venous valves. The device is positioned externally around the vein at

the site of the defective valve. The device provides an external, constricting force to the vein to partially flatten the vein to an oval shape to help restore proper valve operation. Accordingly, one of ordinary skill in the art would not look to Zukowski '755 when faced with designing a method for delivering a biologically active agent to a target type inside a blood vessel. **Second**, claim 74 recites delivering the coiled prosthesis to target site within a blood vessel (as opposed to structure of Zukowski '755 being positioned on the outside a vein) and radially expanding the prosthesis to press against the wall of the blood vessel (as opposed to radially contracting the device of Zukowski '755). More specifically, there is nothing in Zukowski '755 disclosing or suggesting the steps of:

> "delivering a coiled prosthesis to a target site inside a blood vessel of a patient, ... the prosthesis comprising ... a dispensable, biologically active agent within the sleeve interior;
>
> radially expanding the prosthesis from the radially-contracted state to a radially-expanded state so to press the prosthesis against the wall; and
>
> releasing the agent into the blood vessel, the agent passing from the interior, through the material and into the blood vessel.

Rather, the structure of Zukowski '755 surrounds the blood vessel and radially contracts so that any delivery of an agent would pass from the material and into the interior of the device for application to the outer surface of the blood vessel. This is substantially the exact opposite of what is being claimed. Therefore, claim 74 is allowable over the cited art.

4. Claim Rejection-35 USC §103

Claim 11 was rejected under 35 USC 103 (a) as being unpatentable over Razavi '685 in view of McNamara '370.

Dependent **apparatus claim 11**. Claim 11 (parent: claim 38) recites that the adjacent turns touch one another when in the radially-expanded state. Claim 38 recites a coiled sleeve of porous material. In contrast, McNamara is designed to create tight junctions between the adjacent coils to prevent ingrowth of tissue. Therefore, it would have been contrary to the teachings of McNamara to create a stent that encourages tissue ingrowth as could the invention of claim 11. Accordingly, claim 11 is allowable over the cited art.

5. Claim Rejection -35 USC §103

Claim 46 was rejected under 35 USC 103 (a) as being unpatentable over Zukowski '755 in view of Kropf '849, Ragheb '904 and McNamara '370.

Dependent **method claim 46.** Claim 46 (parent: claim 43) recites that "the radially expanding step is carried out with a prosthesis comprising turns which touch one another when in the radially-expanded state." However, there is nothing in the cited art which would have made it obvious to one of ordinary skill in the art to modify the externally applied, constricting device of Zukowski '755 based upon the hollow body nitinol stent of McNamara '370. The two structures are used for completely different purposes so that the construction of the hollow body stent of McNamara '370 would provide no meaningful guidance to modify the device of Zukowski '755 to arrive at the presently claimed invention.

6. Claim Rejections-35 USC §103

Claims 43 and 106 were rejected under 35 USC 103 (a) as being unpatentable over Zukowski '755 in view of Kropf '849 and Hanson '352.

Independent method claim 43. **First**, as mentioned above, Zukowski '755 is directed to a device used to repair defective venous valves. The device is positioned externally around the vein at the site of the defective valve. The device provides an external, constricting force to the vein to partially flatten the vein to an oval shape to help restore proper valve operation. Accordingly, one of ordinary skill in the art would not look to Zukowski '755 when faced with designing a method for delivering a biologically active agent to a target site inside a blood vessel. **Second,** claim 43 recites delivering the coiled prosthesis to target site inside a blood vessel (as opposed to structure of Zukowski '755 being positioned on the outside a vein) and radially expanding the prosthesis to press against the wall of the blood vessel (as opposed to radially contracting the device of Zukowski '755). **Third,** there is nothing in the art suggesting "selecting a coiled prosthesis comprising a coiled body having longitudinally extending side members and cross members connecting said side members, ... ". Kropf '849 discloses a solid metal stent having apertures to permit tissue ingrowth through the apertures. Therefore, Kropf '849 teaches away from using "a material extending along a coiled path along the entire coiled body ..." because doing so could hinder or prevent the desired tissue ingrowth. In addition, there is nothing in Zukowski '755 suggesting that the additional structural characteristics provided by the ladder-type stent of Kropf '849 would have been useful or desirable. Therefore, the art lacks the appropriate teaching or suggestion to substitute the metal stent of Kropf '849 for the coiled wires of Zukowski '755. Therefore, claim 43 is allowable over the cited art.

Dependent **method claim 106.** Claim 106 (parent: claim 43) recites that the selecting step comprises choosing an agent comprising an NO generator. Zukowski '755 is concerned with restoring

proper operation to a defective venous valve so that there would have been no reason to modify Zukowski '755 in light of the teaching of Hanson '352 as suggested by Examiner. Claim 106 is therefore allowable over the cited art.

7. Claim Rejections-35 USC §103

Claims 105 and 107 were rejected under 35 USC 103 (a) is being unpatentable over Razavi '685 in view of Hanson '352.

Dependent **apparatus claim 105**. Claim 105 (parent: claim 38) recites that the agent comprises NO created within the sleeve interior by an NO generator.

It has been found that nitric oxide (NO) is useful to reduce restenosis. See Exhibit A, John B. Cooke MD PhD, Nitric Oxide and Restenosis, A Report For Vascular Architects, Sept. 16, 2002. At room temperature NO is a gas. NO has, however, a short half-life in the body. The testing discussed at Exhibit B, Junghan Yoon, et al, Local Delivery of Nitric Oxide from an Eluting Stent to Inhibit Neointimal Thickening in a Porcine Coronary Injury Model, Yonsei Med J, Vol. 43, No. 2, pp.242-251, 2002, discloses that coating stents with an NO generator incorporated into a polymer was not an effective method for delivery of NO. "However, this sodium nitroprusside-eluting stent failed to reduce chronic neointima thickening in the porcine coronary stent injury model." Exhibit B, page 250. It is believed that the reason for this ineffectiveness in preventing restenosis is due to the manner of the conventional delivery of NO: coating a metal stent with an NO generator.

In contrast, applicants have found through experimentation that a prosthesis, for use inside a blood vessel, made according to claim 105 (Exhibit C, aSpire® covered stent Product Literature) released NO at a therapeutically effective level for over 60 days. It is believed that this extended-length release period is due to the containment of a therapeutically effective amount of the NO generator within a sleeve interior, the sleeve interior being defined by a sleeve of porous material. See Exhibit D (declaration of Kirti Kamdar describing the experiment) and Exhibits E and F (plots of NO vs. time for the experiment).

Applicants are not taking the position that the use of an NO generator per se is new. Rather, the art fails to recognize that there would be an advantage in using an NO generator within the interior of a sleeve of porous material as presently claimed. The evidence of record, discussed above, teaches away from coating stents with an NO generator (because it is ineffective). Further, there is nothing in the art that teaches or suggests using a biologically active agent comprising NO created within the sleeve interior by an NO generator, the agent being "dispensable from the sleeve interior, through the

inner surface, through the material, out of the outer surface and into a blood vessel of a patient." By virtue of placing the NO generator within the sleeve interior, a therapeutically effective amount of NO generated by the NO generator can be delivered to the blood vessel over a therapeutically effective time period. The art simply lacks any recognition of this aspect of the invention.

Dependent **method claim 107**. Claim 107 (parent: claim 74) recites that the delivering step comprises choosing an agent comprising NO created within the sleeve interior by an NO generator.

It has been found that nitric oxide (NO) is useful to reduce restenosis. See Exhibit A, John B. Cooke MD PhD, Nitric Oxide and Restenosis, A Report For Vascular Architects, Sept. 16, 2002. At room temperature NO is a gas. NO has, however, a short half-life in the body. The testing discussed at Exhibit B, Junghan Yoon, et al, Local Delivery of Nitric Oxide from an Eluting Stent to Inhibit Neointimal Thickening in a Porcine Coronary Injury Model, Yonsei Med J, Vol. 43, No. 2, pp.242-251, 2002, discloses that coating stents with an NO generator incorporated into a polymer was not an effective method for delivery of NO. "However, this sodium nitroprusside-eluting stent failed to reduce chronic neointima thickening in the porcine coronary stent injury model." Exhibit B, page 250. It is believed that the reason for this ineffectiveness in preventing restenosis is due to the manner of the conventional delivery of NO: coating a metal stent with an NO generator.

In contrast, applicants have found through experimentation that delivering a biologically active agent, comprising NO created by an NO generator, to a target site inside a blood vessel by releasing the agent from the interior of a coiled sleeve of material, through the material and into the blood vessel according to claim 107, released NO at a therapeutically effective level for over 60 days. It is believed that this extended-length release period is due to the containment of a therapeutically effective quantity of the NO generator within the sleeve interior. See Exhibit D (declaration of Kirti Kamdar describing the experiment) and Exhibits E and F (plots of NO vs. time for the experiment).

Applicants are not taking the position that the use of an NO generator per se is new. Rather, the art fails to recognize that there would be an advantage in using an NO generator within the interior of a sleeve of material as presently claimed. The evidence of record, discussed above, teaches away from coating stents with an NO generator (because it is ineffective). Further, there is nothing in the art that teaches or suggests using a biologically active agent comprising NO created within the sleeve interior by an NO generator, the agent being "dispensable from the sleeve interior, through the inner surface, through the material, out of the outer surface and into a blood vessel of a patient." By virtue of placing the NO generator within the sleeve interior, a therapeutically effective amount of the NO generator can be carried by the prosthesis for delivery of a therapeutically effective amount of NO to

the blood vessel over a therapeutically effective time period. The art simply lacks any recognition of this aspect of the invention.

The dependant claims not specifically referred to above are directed to specific novel subfeatures of the invention and are allowable for that reason as well as by depending from novel parent claims.

In light of the above remarks and amendments to the claims, applicants submit that the application is in the condition for allowance and action to that end is urged. If the Examiner believes a telephone conference would aid the prosecution of this case in any way, please call the undersigned at (650) 712-0340.

Respectfully submitted,

Dated: 29 July 2003

James F. Hann
Registration No. 29,719

HAYNES BEFFEL & WOLFELD LLP
P.O. Box 366
Half Moon Bay, California 94019
Phone: 650-712-0340
Fax: 650-712-0263

APPENDIX

- Exhibits: A: John B. Cooke MD PhD, *Nitric Oxide and Restenosis, A Report For Vascular Architects*, Sept. 16, 2002.

B: Junghan Yoon, et al, *Local Delivery of Nitric Oxide from an Eluting Stent to Inhibit Neointimal Thickening in a Porcine Coronary Injury Model,* Yonsei Med J, Vol. 43, No. 2, pp.242-251, 2002.

C: aSpire® covered stent Product Literature
D: Declaration of Kirti Kamdar
E: Elution Data (the results of Groups 1 and 2 plotted separately)
F: T 1/2 (single plots for Group 1 and 2 plus a best-fit curve)

- Supplemental Amendment (copy) filed by facsimile on 22 May 2003

EXHIBIT A

Nitric Oxide and Restenosis

John P. Cooke MD PhD

A Report for Vascular Architects
September 16, 2002

Nitric Oxide and Restenosis

Restenosis: The Bane of Angioplasty.

Balloon angioplasty is demonstratively effective in the treatment of hemodynamically significant vascular lesions, and is useful in relieving symptoms secondary to obstructive disease. However, the therapeutic utility of angioplasty is limited by re-narrowing of the vessel lumen. Acutely (minutes to days after the procedure), this re-narrowing is due to elastic recoil, and to thrombosis. Chronically(2-6 months), the re-narrowing is due to myointimal hyperplasia as well as adventitial fibrosis and negative remodeling(Fig1). Thrombosis has been reduced by more effective anti-platelet and anti-thrombotic regimens, and elastic recoil and negative remodeling have been eliminated by vascular stents. However, the problem of myointimal hyperplasia remains, and causes significant in-stent restenosis in about 20% of cases. New drug eluting stents may eliminate this remaining problem, and early experience with immunosuppressive agents taxol and sirolimus have been extremely encouraging. Another class of agents which may be equally useful in combination with a drug delivery stent is represented by the NO donors. The following discussion reviews the evidence supporting development of this alternative therapeutic approach.

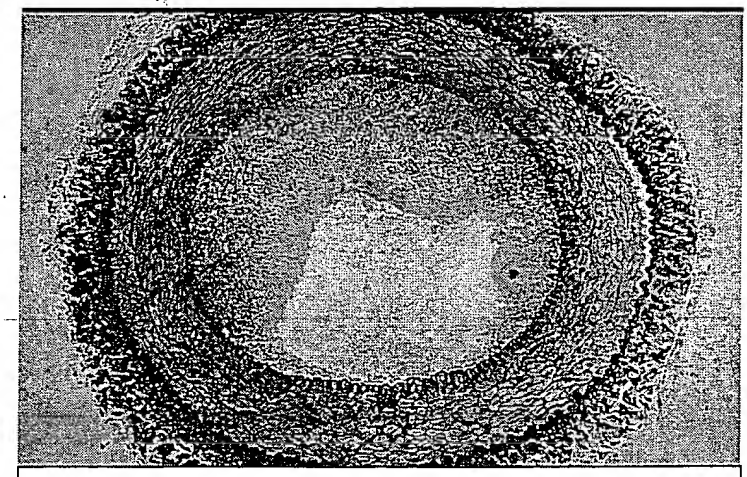

Fig 1. Rabbit iliac artery 4 weeks after angioplasty showing lesion of myointimal hyperplasia.

The Endothelium and Nitric Oxide

In 1998, three American scientists won the Nobel Prize in Medicine or Physiology for their discovery and characterization of a potent endogenous vasodilator elaborated by the endothelium. This endothelium-derived relaxing factor was first described by Robert Furchgott in a citation classic published in 1980(1). Subsequently, Louis Ignarro provided evidence that nitric oxide (NO) was the indentity of this relaxing factor (2). Approximately 10 years before Furchgott's discovery, Ferid Murad had shown that

exogenous nitrovasodilators, such as sodium nitroprusside and nitroglyerin, cause vasodilation by stimulating soluble guanylate cyclase to produce cyclic guanosine monophosphate (cGMP; 3). Ignarro documented that the endothelium derived relaxing factor behaved in the same way. These discoveries had significant ramifications in cardiovascular biology (and in other fields as well).

Endothelium derived NO is derived from the metabolism of L-arginine by NO synthase (NOS). There are three isoforms of NOS: eNOS (the endothelial isoform), nNOS(the neuronal isoform), and iNOS(the inducible isoform, first described in inflammatory cells). These isoforms are not entirely cell specific, eg. eNOS is also found in platelets, and iNOS can be induced in most cells exposed to inflammatory cytokines (4).

In addition to inducing vasodilation, NO elaborated by the endothelium can inhibit platelet adhesion and aggregation; reduce leukocyte adherence and infiltration into the vessel wall; and suppress the proliferation and migration of vascular smooth muscle cells(5). For these reasons, NO has been described as an anti-atherogenic molecule(6).

Nitric oxide and Vascular Structure

Indeed, enhancing the production of vascular NO by administration of the NO precursor (L-arginine) has been shown to inhibit atherosclerosis in mouse and rabbit models(7-9). Furthermore, oral administration of L-arginine has been shown to increase vascular NO synthesis after balloon angioplasty, improve vascular relaxation, and to inhibit restenosis in rat and rabbit models(10-12). Presumably, in this case the NO is derived from iNOS in the injured vessel wall, as angioplasty removes the endothelial source of NOS.

Similar effects have been achieved by transiently transfecting the vessel wall with a plasmid construct encoding eNOS, so as to generate more NO locally. In the rat carotid artery, eNOS gene transfer using lipofection or adenoviral techniqe increased vasular NO elaboration, increased vascular cGMP levels, reduced proliferation of vascular cells, and reduced restenosis after vascular injury (13,14). Similarly, transfection of the injured vessel wall with an adenoviral construct encoding human iNOS inhibits myointimal hyperplasia in rat and pig models of balloon angioplasty. In vivo iNOS gene transfer to injured rat carotid arteries, resulted in a near complete (>95%) reduction in neointima formation even when followed longterm out to 6 weeks post-injury(15). This protective effect was reversed by the continuous administration of an iNOS selective inhibitor L-N6-(1-iminoethyl)-lysine(15). In an animal model more relevant to human vascular healing, iNOS gene transfer (5 x 10(8) PFU/pig) to injured porcine iliac arteries in vivo was also efficacious, reducing intimal hyperplasia by 52%(15). Similar results have been obtained in porcine coronary arteries after angioplasty using the Infiltrator catheter for intramural administration of an adenoviral construct encoding human eNOS (16).

Conversely, genetic or pharmacological inhibition of vascular NO elaboration accelerates atherosclerosis and restenosis. In the hypercholesterolemic NZW rabbit, chronic administration of NOS antagonists increased plaque area and thickness(17). In the eNOS deficient mouse, atherosclerosis is accelerated. When this mouse is bred with the

hypercholesterolemic apo E deficient mouse, atherosclerosis is severe, and even results in atherosclerotic aortic aneurysms(18).

Antagonism of NO synthase by administration of L-NAME exacerbates myointimal hyperplasia after experimental angioplasty (19). To conclude, endogenous NO produced by the vessel wall, causes vasodilation, inhibits platelet and leukocyte adhesion, suppresses cellular proliferation and migration, and prevents vascular lesion formation. Similar observations have been made with exogenous NO donors as described below.

NO donors and Platelet Inhibition

There is a substantial body of data indicating that NO donors suppress platelet adherence to the vessel wall, and inhibit platelet aggregation, at doses that are clinically relevant(20). The effect of NO donors is mediated by activation of soluble guanylate cyclase within the platelet(21). The subsequent production of cGMP leads to the activation of cGMP dependent kinases which phosphorylate proteins such as vasodilator-stimulated phosphoprotein (VASP; 22). In platelets, VASP and VASP phosphorylation have recently been demonstrated to be involved in the inhibition of agonist-induced platelet aggregation and, in particular, integrin $\alpha_{IIb}\beta_3$ activation(23). Because platelet aggregation participates in thrombosis at the time of balloon angioplasty, NO-induced platelet inhibition maintains lumen patency immediately after injury. Furthermore, platelets aggregating at the site of vascular injury release growth factors known to activate vascular smooth muscle migration and proliferation such as platelet-derived growth factor (PDGF; 24). Accordingly, by inhibiting platelet adherence and aggregation at the site of vascular injury, NO further inhibits the proliferative vascular response to injury.

NO donors and Leukocyte Infiltration

The mechanism by which NO inhibits monocyte adhesion is probably multifactorial. NO can inhibit monocyte adhesion to the endothelium, mediated by cGMP modulation of adhesion signaling(25). However, NO also downregulates the endothelial expression of monocyte chemotactic protein-1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1), which play critical roles in monocyte-vessel wall interaction(26-28). By contrast, inhibition of NO synthase increases the expression of endothelial proteins required for monocyte adhesion(27). Studies from our laboratory and others implicate the existence of an oxidant-sensitive transcriptional pathway that activates the expression of VCAM-1 and MCP-1(27-29). Endogenous NO or exogenous NO donors, inhibit endothelial elaboration of superoxide anion, reduce the activity of NFκB, suppress the stimulated expression of VCAM-1 and MCP-1, and reduce endothelial adhesiveness for monocytes. NO may exert these effects in part by inhibiting the generation of superoxide anion by oxidative enzymes(30).

NO Donors and Vascular Smooth Muscle Migration and Proliferation

A major component of in-stent restenosis is the migration and proliferation of vascular smooth muscle cells(Fig 2). NO inhibits vascular smooth muscle cell proliferation(31). NO inhibits smooth muscle mitogenesis at distinct points in the cell cycle by cGMP-dependent(late G1 phase) and –independent(S phase) mechanisms(32). In one study,

vascular smooth muscle cells (VSMCs) were transduced with an adenoviral vector encoding eNOS (AdeNOS) or beta-galactosidase (Ad beta Gal). eNOS expression was detected in transduced VSMCs and cGMP levels were increased. These effects were associated with a delay in cell cycle progression and upregulation of p27 and p21(33).

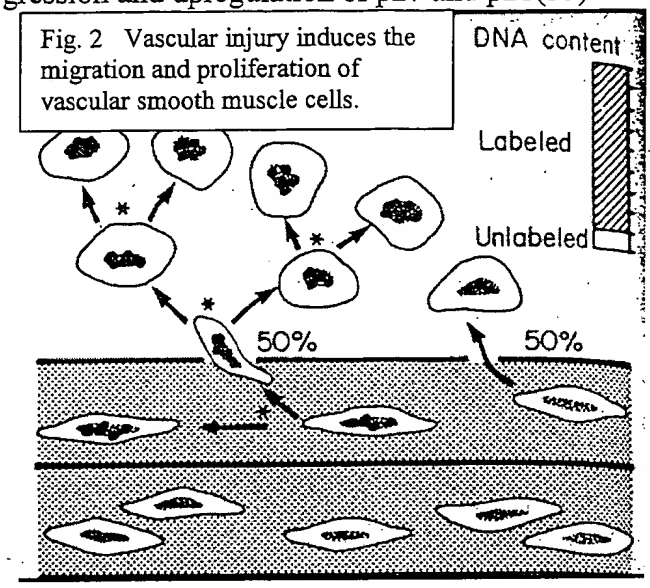

Fig. 2 Vascular injury induces the migration and proliferation of vascular smooth muscle cells.

Similarly, exogenous NO donors inhibit vascular smooth muscle proliferation. DETA/NO, a well-characterized NO donor of low MW (163 Da) with a half-life for NO release of 20 hours suppressed vascular smooth muscle cell proliferation in culture completely but without evidence of toxicity (34).

After vascular injury the migration of VSMCs from the media to the intimal space is an important component of the process of restenosis. The effect of NO donors on this process has been studied in vitro using migration assays. In one such study, the migration of primary cultured VSMCs (derived from canine femoral artery) was assessed and related to cGMP levels(35). The stable analogue of cGMP, 8-Bromo-cGMP, inhibited VSMC migration. When inducible nitric oxide synthase (iNOS) was induced by 24-hour preincubation with lipopolysaccharide and interleuken-1beta, basal migration decreased and cGMP production increased. Under these conditions, insulin further reduced VSMC migration, an effect which was blocked by the nitric oxide synthase inhibitor LNMMA, as well as by 1-H-1[1,2,4]oxadiazolo-[4, 3a]quinoxolin-1-one, a selective inhibitor of guanylate cyclase. These data suggest that NO-induced increases in cGMP reduce VSMC migration. However, there is evidence that NO may elicits opposite effects on cell migration and proliferation in primary versus subcultured cells (36)

NO and Apoptosis

NO induces apoptosis in vascular smooth muscle cells (37). In cultured vascular smooth muscle cells, addition of the NO donor molecules S-nitroso-N-acetylpenicillamine or sodium nitroprusside to VSMC dose-dependently induced apoptosis as documented by DNA laddering and quantified by analysis of cellular chromatin morphology. The mediator role of the guanylate cyclase signaling pathway in NO-induced apoptosis was evidenced by induction of apoptosis by the 8-bromo-cGMP analogue; potentiation of NO-induced apoptosis by cGMP-specific phosphodiesterase inhibition; and prevention of NO-induced apoptosis by inhibition of the cGMP-dependent protein kinase 1 alpha.

By contrast, NO donors have anti-apoptotic effects in cultured endothelial cells (38), suggesting that the effect of NO as a modulator of apoptosis is cell-specific and dependent on the presence of certain cytokines, growth factors, or oxidative stress (39).

A rapidly emerging body of evidence suggests that vascular remodeling and lesion formation are determined in large part by the balance between cell growth and cell death by apoptosis(40,41). NO beneficially modulates both. By suppressing vascular smooth muscle cell proliferation, and by increasing apoptosis of VSMCs as well as infiltrating cells (see below), NO reduces the accumulation of cells in the intimal space. Furthermore, by reducing endothelial cell apoptosis, NO assists in the process of re-endothelialization of the wounded vascular segment.

Endogenous and exogenous NO suppresses the accumulation of inflammatory cells after vascular injury. Immunohistochemical studies document the activation of iNOS in the intimal macrophages and vascular smooth muscle cells of human atherosclerotic plaque(42). In the presence of superoxide anion, which is produced under these conditions, the product of iNOS is quickly transformed into peroxinitrite anion, a highly reactive free radical(43) which itself is cytotoxic and may also induce apoptosis by causing DNA strand fragmentation(44). Both NO or peroxynitrite anion could induce apoptosis of vascular smooth muscle cells(37,41).

Exogenous NO donors can induce apoptosis of inflammatory cells in plaque. In atheromatous aortic segments from hypercholesterolemic NZW rabbits, ex vivo addition of sodium nitroprusside (10uM) to the medium caused a time-dependent increase in apoptosis of vascular cells (largely macrophages) in the intimal lesion(45)

NO Donors and Restenosis

As documented by the in vitro studies discussed above, NO is a pleiotropic molecule with effects on multiple processes involved in restenosis. Accordingly, it is not surprising that in all animal models of vascular injury, the great majority of investigators have found that NO donors administered systemically or locally inhibit restenosis. NO donor molecules of several structural classes reduced intimal thickening in rabbits, pigs, mice, and rats(46-48). Inhaled NO(80 ppm NO for 14 days) inhibited restenosis in rat carotid arteries without causing any hemodynamic changes(49).

An interesting new approach has been to combine NO donors with other agents that may have utility in the immediate or chronic setting of vascular injury. In rat and mouse models of myointimal hyperplasia, oral administration of NO-NSAIDS or NO-aspirin formulations have been shown to inhibit restenosis, whereas the parent NSAID or aspirin had little or no effect(50,51). In these studies the anti-restenotic effect was correlated with reductions in vascular proliferation in the injured segment, and with plasma levels of nitrogen oxides. Another advantage of attaching an NO moiety to the NSAID or aspirin was a reduction in gastric ulceration, presumably due to cytoprotective effects and/or increased gastric blood flow produced by the NO released in the stomach. In one of these studies, the NO-releasing aspirin derivative (NCX-4016) reduced the degree of restenosis after balloon angioplasty in low-density lipoprotein receptor-deficient mice. This effect was associated with reduced vascular smooth muscle cell (VSMC) proliferation and macrophage deposition at the site of injury (51).

Local administration of NO donors or the NO precurson L-arginine has also been accomplished using channeled balloon catheters that permit the vessel wall to be bathed

by the agent during angioplasty. In the balloon injury model, vessels are denuded of endothelium, so that endothelial generation of NO from L-arginine is not possible initially. However, in the injured vessel wall, NO is produced by other cells such as proliferating vascular smooth muscle cells and infiltrating monocytes. Here, inducible NO synthase is responsible for NO production and L-arginine becomes rate-limiting(52). Indeed, it has been found that smooth muscle cells in the neointima express inducible NO synthase as early as 1 day after balloon catheter injury and this expression persists for up to 14 days(53). The local expression of iNOS inhibits platelet adherence and aggregation at the injured site; indeed systemic or adventitial application of NOS antagonists increases platelet adhesion at the injured site(54).

Notably, a single intramural administration of an NO donor or the NO precursor can have long-lasting effects on vascular structure and reactivity after balloon angioplasty. A single intraluminal administration of L-arginine has been shown to cause a sustained enhancement of vascular NO generation in the injured segment, resulting in improved vasomotion and inhibition of lesion formation(55). In a subsequent study, hypercholesterolemic New Zealand White rabbits underwent iliac artery angioplasty and a local drug delivery catheter was introduced into both iliac arteries to deliver either L-arginine (800 mg/5 mL) or saline(56). Intramural administration of radioactively labeled L-arginine led to significantly higher counts in comparison to the contralateral segment for up to 1 week after delivery; this was associated with significantly higher NO levels in the L-arginine–treated segments. For a prolonged period after this single administration(2-4 weeks), monocyte binding to the injured segment was significantly decreased by treatment with L-arginine, and there was a 9-fold greater number of apoptotic cells in the vessel wall.

Similar effects have been achieved with local delivery of NO donors. Rolland et al investigated the therapeutic effect of angioplasty with local drug delivery (LDD) of the NO-donor molsidomine in the superficial femoral arteries of atherosclerotic swine(57). Atherosclerotic Pietrin swine underwent angioplasty with delivery of 4 mg molsidomine in 2 ml of vehicle using a channelled balloon angioplasty catheter. In comparison to vehicle control, 24 hours after the injury, there was about a 60% reduction in proliferating vascular smooth muscle cells in the NO donor treated vascular segments as demonstrated by staining for PCNA-positive nuclei. At 5 months, molsidomine treated vessels, manifested increased compliance and reduced impedance. Histomorphometry revealed less restenotic intimal thickening and a greater lumenal diameter(by about 35%) in molsidomine-treated versus placebo-treated vessels.

Similar results were obtained by Kalinowski et al(58). New Zealand white rabbits underwent balloon dilation of both common iliac arteries to induce arterial stenosis. Four weeks later, one stenotic iliac artery was simultaneously dilated and received local application of L-arginine (210 mg/mL, n = 7), r-hirudin (0.5 mg/mL, n = 8), or molsidomine (0.2 mg/mL, n = 8) with a channeled balloon catheter. On the contralateral side, 0.9% saline was injected as a control. Six weeks after local treatment, vessels were harvested, and computerized morphometric and immunohistologic analyses were performed. In comparison to vehicle treated segments, those treated with L-arginine,

molsidomine or hirudin manifested significant reductions in myointimal hyperplasia (53%, 43%, and 20% respectively) in comparison to control. Immunohistologic findings showed a significant reduction of macrophages and proliferating cells in the neointima after local application of L-arginine.

Similar findings have been observed with the S-nitrosothiol class of NO donors. In one study, S-nitroso-bovine serum albumin, or a polythiolated form of bovine serum albumin modified to carry several S-nitrosothiol groups, were administered intraluminally to the injured rabbit femoral artery. The single administration of the nitrosylated peptides increased vascular NO generation, increased tissue cGMP, inhibited platelet aggregation and deposition at the site of injury, and significantly reduced myointimal hyperplasia assessed at 2 weeks following the injury(59). These effects were directly related to the amount of NO released at the site of vascular injury, with the polythiolated form being more efficacious.

The diazeniumdiolated derivative of albumin (D-BSA), is a derivatized protein containing 22 diazeniumdiolate groups per molecule with a 20-day half-life for NO release. Intrapericardial administration of D-BSA reduced coronary artery myointimal hyperplasia at two weeks by 50% in comparison to underivatized albumin in a swine model of balloon angioplasty(60). In addition, positive remodeling was noted, with a larger total vascular cross-sectional area. There was no systemic effect of this regional application of NO-donor. Specifically, the regional administration of an NO donor did not affect heart rate or blood pressure. Nor were any histological changes noted in the pericardium or myocardium(60). Similarly, periadventitial exposure of rat iliofemoral arteries to a gel containing an NO-releasing diazeniumdiolate during and after balloon injury produced a marked reduction of intimal hyperplasia 2 weeks after vascular injury(61).

Incorporation of NO donors into polymers may be useful for device applications. Nitric oxide donors with different half-lives have been covalently incorporated into photopolymerized polyethylene glycol hydrogels(62). Under physiological conditions, NO was produced by these hydrogels over periods ranging from hours to months, depending upon the polymer formulation. The NO-releasing materials successfully inhibited smooth muscle cell growth in culture. Platelet adhesion to collagen-coated surfaces was also inhibited following exposure of whole blood to NO-producing hydrogels.

To assess the effect of a NO-eluting stent on reducing neointimal thickening in a porcine coronary artery stent injury model, sodium nitroprusside (SNP), a NO donor, was incorporated into polyurethane polymer and coated onto metallic coil stents, and two types of stents with thin and thick barrier coatings were characterized. In vitro studies revealed that the SNP-coated stents released NO in a controlled manner for up to 4 weeks. In the in vivo studies, an increase in vascular cGMP levels at the site of the stent implantation was demonstrated for up to 14 days. The neointimal area at 28 days was not diminished, however, by NO eluting stents. The lack of an effect may have been due to inadequate tissue levels, or insufficient duration of release. (63). Similar results were

obtained using a tantalum coil coronary stents covered with an NO donor(64).

Clinical Investigations into NO and Restenosis

An intriguing study by Fukomoto and colleagues indicated that the ability of the vascular wall to produce NO after angioplasty correlated with less risk for restenosis(65). In 23 consecutive patients, the ability of the vessel wall to vasodilate in response to intra-coronary L-arginine infusion was assessed 18 hours after angioplasty (at a point in time were iNOS should be induced locally at the site of the vascular injury). L-arginine infusion induced a greater vasodilation at the angioplasty site, than at a distal uninjured segment. Notably, the magnitude of the vasodilator response to L-arginine correlated with the coronary artery diameter 3 months after PTCA. These results suggest that augmented NO production after PTCA may protect against the development of coronary restenosis. The authors surmised that "treatment that enhances local NO production may be clinically useful in preventing restenosis after PTCA".

Further proof of concept was obtained in the ACCORD study(66). This was a prospective multicenter, randomized trial, in which 700 stable coronary patients scheduled for angioplasty received direct NO donors (infusion of linsidomine followed by oral molsidomine) or oral diltiazem. Treatment was started before angioplasty and continued until 12 to 24 hours before follow-up angiography at 6 months. Pretreatment with an NO donor was associated with a modest improvement in the immediate angiographic result compared with pretreatment with diltiazem (minimum luminal diameter, 1.94 versus 1.81 mm; P = .001); this improvement was maintained at the 6-month angiographic follow-up (minimal lumen diameter, 1.54 versus 1.38 mm; P = .007). Restenosis, defined as a binary variable (> or = 50% stenosis), occurred less often in the NO donor group (38.0% versus 46.5%; P = .026). Combined major clinical events (death, nonfatal myocardial infarction, and coronary revascularization) were similar in the two groups (32.2% versus 32.4%). The investigators concluded that treatment with the NO donor was associated with a modest improvement in the long-term angiographic result after angioplasty although there was no effect on clinical outcome. The improved angiographic result related predominantly to a better immediate procedural result, because late luminal loss did not differ significantly between groups. The modest effects of NO donors on restenosis in this trial may be due to the fact that the NO donor was administered systemically, rather than at high local concentrations.

Support for this view was provided by a recent study examining the effect of intramural administration of L-arginine on in-stent restenosis(67). To determine whether intramural administration of L-arginine reduces intimal thickening after coronary stent deployment in humans, 50 patients with native coronary artery disease who received a single Palmaz-Schatz stent were enrolled in this pilot study. Patients were randomized to receive L-arginine (600 mg/6 ml) or saline (6 ml) delivered locally via the Dispatch catheter (Scimed) over 15 minutes. Serial angiography and intravascular ultrasound examinations

(motorized pull-back at 0.5 mm/s) were performed before and after the procedure, and at 6-month follow-up. At 6 months, neointimal volume in the L-arginine group was reduced by 36%.

Platelets are activated in patients undergoing PTCA as demonstrated by measurement of surface expression of P-selectin and glycoprotein IIb/IIIa in the platelets derived from coronary sinus vein blood samples despite systemic treatment with aspirin, glyceryl trinitrate, and heparin. Intracoronary infusion of the NO donor GSNO, starting 10 min before PTCA, significantly inhibited the PTCA-induced increase in platelet surface expression of P-selectin and glycoprotein IIb/IIIa without altering blood pressure(68). Thus local administration of NO donors can also inhibit platelet activation that occurs in the setting of angioplasty. This evidence is pre-clinical studies, local administration of NO donors to the injured vessel wall should also reduce acute thrombosis after angioplasty and stenting.

Summary

Nitric oxide is a potent vasodilator, and has significant effects on vascular structure by virtue of its ability to suppress vascular smooth muscle proliferation, reduce platelet adherence and aggregation, inhibit leukocyte infiltration, increase apoptosis of proliferating and inflammatory cells, and enhance endothelial regeneration. Local enhancement of vascular NO activity at the site of vascular injury may be an alternative therapeutic strategy to inhibit thrombosis and restenosis after balloon angioplasty and stenting.

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EXHIBIT 6

Yonsei Medical Journal
Vol. 43, No. 2, pp. 242 - 251, 2002

Original Article

Local Delivery of Nitric Oxide from an Eluting Stent to Inhibit Neointimal Thickening in a Porcine Coronary Injury Model

Junghan Yoon[1], Chiung-Jen Wu[2], James Homme[3], Ronald J. Tuch[3], Rodney G. Wolff[3], Eric J. Topol[4], and A. Michael Lincoff[4]

[1]Department of Internal Medicine, Yonsei University Wonju College of Medicine, Wonju, Korea;
[2]Chang-Gung Memorial Hospital, TA-PEI Rd, Niao-Sung Hsiang, Kaohsiung Hsien, Taiwan;
[3]Medtronic Inc., 710 Medtronic Parkway, Minneapolis, MN 55432-5604, U.S.A.;
[4]Department of Cardiology, F25, The Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195-5066, U.S.A.

To assess the effect of a NO-eluting stent on reducing neointimal thickening in a porcine coronary artery stent injury model, sodium nitroprusside (SNP), a NO donor, was incorporated into polyurethane (PU) polymer and coated onto metallic coil stents, and two types of stents with thin and thick barrier coatings were characterized. *In vivo* biological activity of the NO-eluting stents was assessed by measurement of coronary arterial cGMP levels in 32 pigs/64 arteries at days 1, 2, 7 and 14. Morphometric analyses were performed in 16 pigs to determine the effect of NO-eluting stents on neointimal hyperplasia 28 days following arterial injury. The SNP-coated stents released NO in a controlled manner for up to 4 weeks in the *in vitro* experiments and an increase in local tissue cGMP levels was demonstrated for up to 14 days. The neointimal area at 28 days was not diminished, however, by NO eluded from either stents of thin or thick barriers (control bare stent - 0.66 mm^2, control PU stent - 0.68 mm^2, SNP-PU thin coating stent - 0.78 mm^2, SNP-PU thick coating stent - 0.85 mm^2; all p=NS). In conclusion, the SNP-coated polymer stent exerted a local biological effect on the arterial wall, with sustained elevation of cGMP level. Although local delivery of NO from this device did not reduce neointimal hyperplasia in this porcine model, this polymer-coated stent might be a promising tool for administration of other agents that may modify the reparative tissue responses leading to restenosis.

Key Words: Nitric oxide, restenosis, coronary artery disease, angioplasty

Received November 14, 2001
Accepted February 14, 2002

Reprint address: requests to Dr. A. Michael Lincoff, Department of Cardiology, F25, The Cleveland Clinic Foundation, 9500 Euclid Ave, Cleveland, OH 44195-5066, U.S.A. Tel: +1-216-444-2367, Fax: +1-216-444-8050, E-mail: LINCOFA@CCF.ORG

INTRODUCTION

Local drug delivery is an attractive therapeutic option for the prevention of restenosis, given that restenosis is a localized pathologic process. This technique may allow high local tissue concentrations of drug without side effects of systemic drug administration.[1,2] The tissue reparative processes that lead to restenosis after angioplasty may be sustained for a time frame of days to weeks, and a method of releasing drug for a prolonged period after arterial injury may be needed. A drug-eluting polymer-coating stent is one potential technique to achieve high local tissue concentrations of an effective drug for a prolonged period after arterial injury, in addition to the beneficial effects of the metallic stent on arterial remodeling.

Besides a primary role in regulating the vascular tone,[3] nitric oxide, one of the important products of normal endothelium, has been postulated to reduce vascular lesion formation by inhibiting platelet adhesion,[4] leukocyte adhesion,[5,6] vascular smooth muscle cell migration and proliferation,[7,8] and protein and collagen synthesis of the vascular smooth muscle cells.[9] In several animal models, dietary supplementation of L-arginine, local delivery of a nitric oxide synthetase gene, and local delivery of nitric oxide donors restored endothelial function and reduced neointimal thickening.[10-14]

Endothelial dysfunction has been demonstrated

to last up to 4 to 8 weeks after injury, even after the endothelium is regenerated completely,[15,16] with local deficiency of nitric oxide during that time. A means of prolonged delivery of nitric oxide would likely be required if local administration were to inhibit the neointimal hyperplastic response to arterial injury. In the current study, we used sodium nitroprusside (SNP) as a nitric oxide donor, and impregnated it into a polyurethane (PU) polymer and coated onto a metallic stent. We evaluated the efficacy of this local delivery system on neointimal thickening in the porcine coronary artery stent overexpansion injury model.

MATERIALS AND METHODS

Nitric oxide eluting stents were used for arterial injury and local drug delivery. Sodium nitroprusside (SNP), a nitric oxide donor, was incorporated into a polyurethane (PU) polymer and coated onto tantalum coil wire stents. Characterization of the SNP-PU stent device was performed by an *in vitro* nitric oxide-elution kinetics study. The *in vivo* biological activity of nitric oxide eluted from this stent was assessed by measurement of tissue cyclic GMP levels. Morphometric studies were performed to determine the efficiency of nitric oxide stent on neointimal hyperplasia following arterial injury.

Nitric oxide-eluting stent

SNP releases nitric oxide either spontaneously or in the presence of sulphydryl groups.[17-19] The nitric oxide eluting stent consisted of a 125-μm diameter tantalum wire configured into a 16-mm long balloon-expandable coil stent (Wiktor, Medtronic, Inc., Minneapolis, MN, U.S.A.) and coated with a monolithic matrix of PU (Medtronic proprietary formulation) and SNP covered by PU barrier layer (Fig. 1). A 2% (w/w) solution of PU in tetrahydrofuran was prepared, in which a 3% suspension of SNP was formed. This mixture was sprayed onto the stent wires to form the SNP eluting polymer coating. The ratio of SNP to PU in the base coating was 3:2, with 1.9 - 2.0 mg SNP per stent. This SNP coating was covered with a barrier layer of either - 0.7 mg (thin coating stent)

Fig. 1. Photograph of SNP-PU thick coating stent (7× magnification).

or - 2.2 mg (thick coating stent) of PU. A PU-coated control stent (2.9 mg of PU) was prepared using same method. The thickness of the coating for these stents ranged from - 20 μm for the controls to - 80 μm for the thick barrier coating stents. The polymer coating was demonstrated by microscopy to be sufficiently flexible to allow balloon expansion of the coil wire stent without cracking or peeling from the wire. Stents were sterilized using a conventional ethylene oxide gas technique and hand-mounted on the commercial angioplasty balloons before stent implantation. We tested 4 different stent designs: control-bare stents, control PU polymer stents without impregnation of SNP, SNP-PU thin coating stents, and SNP-PU thick coating stents.

In vitro elution kinetics

The elution kinetics of SNP from sterilized polymer-coated stents were characterized in an *in vitro* system using a colorimetric assay based on the nitrosation of famotidine. Stents were placed in glass vials and immersed in 4.0 ml of phosphate-buffer saline. At time points ranging from two hours to 39 days, aliquots of the elution buffer were taken and SNP concentration measured using a famotidine assay.[20] Famotidine reacts with SNP to give absorbance peaks at 394 and 498 nm. Absorbance was measured at 394 nm and 498 nm by ultraviolet spectrophotometry (model 8452, Hewlett-Packard, Palo Alto, California, U.S.A.) and converted to cumulative elution curves. The absorbance at 394 nm is indicative of "total" SNP. The peak at 498 nm, although smaller, represents

the nitrosated famotidine and is indicative of "active" SNP. SNP concentration was determined by comparison to standard curves generated on the same day.

Animal procedures and study groups

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85 - 23, revised 1996).

Juvenile domestic farm pigs, weighing 20 - 25 kg, were treated with oral aspirin 325 mg preoperatively and daily thereafter until sacrifice. General anesthesia was induced with intramuscular injection of ketamine (22 mg/kg; Fort Dodge Laboratories Inc., Fort Dodge, Iowa) and maintained with inhalation of isoflurane (Abbott Laboratories, Chicago, IL, U.S.A.) during the procedure. The right carotid artery was exposed through a midline neck incision, and a 9 Fr hemostatic arterial sheath was introduced over a guidewire through a carotid arteriotomy. Heparin (300U/kg; Elkins-Sinn Inc., Cherry Hill, NJ, U.S.A.) was administered as a single intravenous bolus. Left and right coronary angiography was performed using an 8 Fr guiding catheter (Hockeystick, Cordis, Miami, FLA, U.S.A.). Based upon the baseline angiography, coronary arterial size was estimated and suitable epicardial sites were chosen to allow deployment of a stent, over expanded by 15 - 20%, using a 3.0 - 4.0 mm angioplasty balloon catheter. Stents were hand-crimped onto the selected balloon catheter and deployment was performed under fluoroscopic guidance over a standard 0.014-inch angioplasty guidewire. Animals were randomly assigned to receive control bare stents, control polymer stents, SNP-PU thin coating stents, or SNP-PU thick coating stents. Two or three coronary arteries per animal underwent stent over-expansion injury using the same stent type in each artery. The balloon was inflated once for 30 seconds with inflation pressure of 8 atm to deploy the stent. Follow-up angiography was performed following stent implantation to confirm adequate stent expansion and vessel patency. The arteriotomy site was ligated and the neck wound closed with continuous interrupted sutures. Animals were kept alive on a standard laboratory chow diet throughout the study period.

For determination of tissue cGMP levels, animals were sacrificed at day 1, 2, 7, or 14 following the stent implantation. Under general anesthesia with intramuscular injection of ketamine (50 mg), 10,000 U of heparin was given intravenously as a bolus injection, and euthanasia was induced with intracoronary injection of potassium chloride (40mEq). The heart was immediately explanted, and peri-arterial fat and connective tissue were carefully removed. Stented and normal coronary arterial segments (about 1 cm in length) proximal and distal to the stented segment of the coronary artery were excised. Harvested specimens were immediately frozen in liquid nitrogen and kept at -70℃ until analysis of tissue cGMP levels.

For the morphometric analysis, follow-up coronary angiography was performed under general anesthesia 28 days after stent implantation. Pigs were euthanized by over-dose of intracoronary injection of potassium chloride and the heart was removed and perfusion-fixed at 70 mmHg for 24 hours with 10% neutral buffered formalin. Stent-containing coronary arterial segments were removed and sectioned at 2-mm intervals perpendicular to the vessel axis. Coronary arterial segments were embedded in paraffin blocks, sectioned and stained with conventional hematoxylin-eosin and Lawson's elastic van Giesson stains.

Determination of coronary arterial cGMP levels

Determination of local arterial cGMP levels was performed to evaluate the biological activity of the stent-based nitric oxide delivery. Stented arteries were divided into proximal, stented, and distal segments. Each segment was minced using a scalpel and homogenized in 750 μL of ice cold 6% trichloroacetic acid using a motorized tissue homogeneizer (VirTis Handishear with 6-mm shaft; VirTis Company, Gardiner, N.Y., U.S.A.). After centrifugation at 10,000 g for 15 minutes at 4℃, pellets were kept for the protein analysis in the refrigerator and supernatants were extracted three times with 1 vol. water-saturated diethylether (Sigma, St. Louis, MO, U.S.A.). The ether portion was totally removed and the water-extracted

portion was lyophilized at - 52℃ in a vacuum state using freeze dry system (Lyph 4.5, LABCONCO Corporation, Kansas City, MO, U.S.A.).

The concentration of cGMP in each supernatant sample was determined using a commercially available cGMP enzyme-immunoassay (EIA) kit (Amersham, Life Science, Chicago, IL, U.S.A.) with the addition of acetylation step to increase the sensitivity. Pellets from the initial homogenization step were digested in 1 ml 0.1 N NaOH overnight at 60℃ to extract protein, which was assayed by using a Pierce BCA kit (Pierce Chemical Co., Rockford, IL, U.S.A.).

Histomorphometric analysis

Morphometric analyses were performed using light microscopy and a computerized digital microscopy algorithm (Image-1/MetaMorph; Universal Imaging Corporation, West Chester, PA, U.S.A.). All coronary segments were qualitatively inspected by observers (J.Y. and W.C.) blinded to study group who assessed for the presence of thrombus and inflammatory cell infiltration and evaluated the depth of arterial injury by each stent struts. In every stented arterial segment, the single histologic section showing the most severe luminal narrowing was used for measurements of lumen area, internal elastic lamina (IEL) area, and external elastic lamina (EEL) area. Medial area was calculated as EEL area minus IEL area. Intimal area was calculated as IEL area minus luminal area. Maximal intimal thickness was measured at each stent wire site, and an injury score at each stent wire site was assigned by the depth of the stent wire disruption of the vessel wall structure: 0 = intact IEL; 1 = IEL lacerated; 2 = IEL and media lacerated; and 3 = EEL lacerated.[21]

Statistical analysis

The Kruskal-Wallis test was used to determine the statistical significance of differences in coronary arterial cGMP levels among 4 different stent groups, and the Man-Whitney test was applied to the 6 sets of 2 group combinations to assess statistical significance.

Continuous variables of morphometric measurements are expressed a mean +/- standard deviation. The ANOVA test was used for comparison of luminal areas, intimal areas and intimal thicknesses among 4 different groups. A p value < 0.05 was considered to be statistically significant. To determine the influence of SNP-PU coated stents on the extent of intimal hyperplasia 28 days after stent implantation over control bare and control PU stents, linear regression curves relating intimal thickness to injury scores were plotted for each treatment group.[21,22] A reduction in the neointimal thickness to arterial injury due to therapy would result in a decrease in the slope or the intercept of this regression relation, or both.[2] Thus, these slope and intercept values serve as end points for comparing study groups. Linear regression analysis for mean neointimal thickness versus mean injury score was performed using arterial segments obtained from four study groups (control bare stents, control PU stents, SNP-PU thin coating stents, and SNP-PU thick coating stents). Three binary variables, SP, S_{S1}, and S_{S2} (value 0 or 1) representing the groups of PU coated stents, SNP-PU thin coating stents or SNP-PU thick coating stents, respectively, and their interaction terms, injury score*S_P, injury score*S_{S1}, and injury score*S_{S2}, were added to the regression equation to evaluate whether any stent coating produced a statistically significant change in slope or intercept. The following multiple regression model was generated:

$$\text{Mean neointimal thickness} = [\text{Slope} + (\alpha_P \times S_P) + (\alpha_{S1} \times S_{S1}) + (\alpha_{S2} \times S_{S2})] \times \text{mean injury score} + \text{Intercept} + (\beta_P \times S_P) + (\beta_{S1} \times S_{S1}) + (\beta_{S2} \times S_{S2}),$$

where α_P, α_{S1}, and α_{S2} are the coefficients of S_P, S_{S1}, and S_{S2} and β_P, β_{S1} and β_{S2} the coefficients of their interactions, injury score*S_P, injury score*S_{S1}, and injury score*S_{S2} estimated by multiple regression. A statistically significant effect of stent coating on the slope of the linear relation between neointimal thickening and injury score was considered if the p value for α_P, α_{S1}, or α_{S2} was < 0.05. A significant effect of stent coating on the intercept of the linear regression curve was considered if the p value for β_P, β_{S1} or β_{S2} was < 0.05.

RESULTS

In vitro SNP-elution kinetics

Elution of SNP started immediately and lasted for at least 2 weeks in the buffered bath with both types of stents (Fig. 2). The SNP-eluting stent with thin barrier coating eluted drug during the first 2 weeks, and while the thick barrier stent eluted drug over approximately 4 weeks. Approximately 85 percent of the total SNP mass in the SNP-PU stents was eluted by 30 days.

Coronary arterial cGMP levels

Stent were implanted in 2 epicardial arteries of each of 33 pigs. A total of 69 stents were used; three stents were embolized to the descending thoracic aorta, and the remaining 66 stents were successfully deployed. One animal died suddenly 2 hours after stent implantation. A total 64 stented coronary arteries were available for tissue cGMP determination.

At 1 day after stent injury (Fig. 3A), cGMP levels in stented segments for all stents were lower than in proximal or distal normal segments in general. cGMP levels in SNP stented segments were significantly higher than those in control stented segments ($p < 0.05$).

At 2 days after stent injury (Fig. 3B), cGMP levels in stented segments remained lower than those in proximal and distal normal segments except for SNP-PU thin coating stents. cGMP levels in stented segments with SNP-PU thick coating stents were significantly lower than those in control stents and SNP-PU thin coating stents. cGMP levels in stented segments with SNP-PU thin coating stent were significantly higher than those in other stents.

At 7 days after stent injury (Fig. 3C), cGMP levels in stented segments of control-bare and control-PU stents remained lower than those in

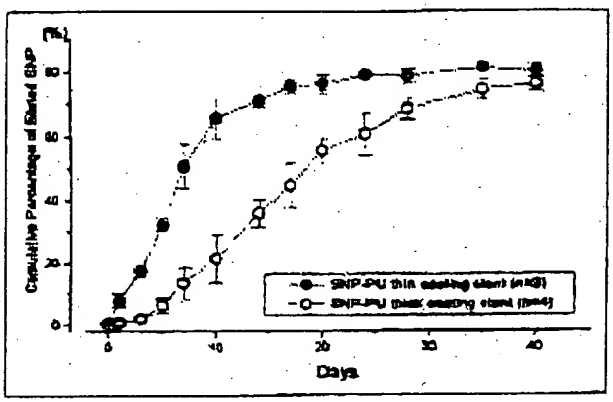

Fig. 2. *In vitro* sodium nitroprusside (SNP) elution kinetic studies using three SNP-PU stents of thin barrier coating and four SNP-PU stents of thick barrier coating. Values are mean ± SE.

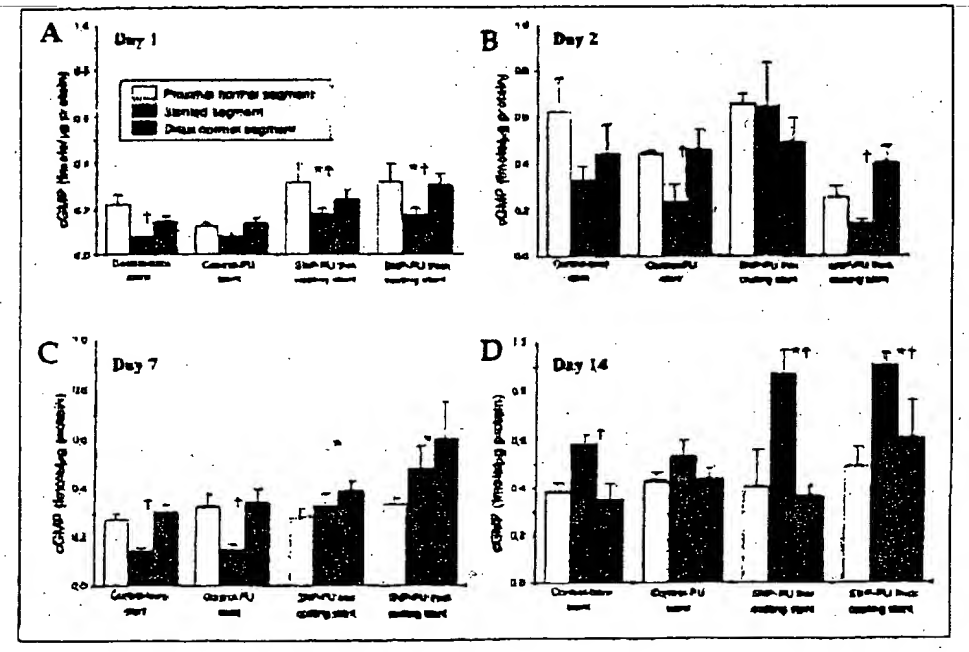

Fig. 3. cGMP levels in stented, proximal, and distal normal segments among 4 treatment groups at (A) day 1, (B) day 2, (C) day 7, and (D) day 14 after stent implantation. The error bar represents 1 standard error. The "*"denotes statistical significance of cGMP levels in stented segments comparing SNP-PU stents with control stents and "†"denotes statistical significance of cGMP levels between stented segments and adjacent non-stented segments. Values are mean ± SE. "*" and "†" $p < 0.05$.

proximal and distal normals ($p < 0.05$). However, cGMP levels in stented segments of both SNP-PU stents were normalized relative to those in proximal and distal normal segments and significantly higher than those in control stented segments ($p < 0.05$).

At 14 days after stent injury (Fig. 3D), all stented segments trended to have higher cGMP levels than proximal and distal normal segments ($p < 0.05$). cGMP levels in stented segments of both SNP-PU stents were significantly higher than those of controls ($p < 0.05$).

Chronic study - neointimal hyperplasia

Stent implantation was attempted in 21 pigs, 3 vessels per pig. A total of 63 stents were used, with 2 stents embolized to the descending thoracic aorta and 1 stent to the left common carotid artery. Thus, 60 stents were successfully implanted in 60 arteries of 21 pigs. Five pigs died 2 to 5 hours after stent implantation, likely due to acute stent occlusion and ischemia (3 in control bare stent, 1 in control PU stent, and 1 in SNP-PU thin coating stent). The remaining pigs survived the 28-day follow-up period. A total of 46 coronary arterial segments in 16 pigs were available for chronic morphometric analysis: 12 in control-bare stent group, 11 in control-PU stent group, 11 in SNP-PU thick coating stent group, and 12 in SNP-PU thin coating stent group.

Histopathologic examination of porcine coronary artery cross-sections 28 days following implantation of the stents showed no inflammatory responses in specimens in which stent struts did not penetrate the external elastic lamina (Fig. 4, left panel). In contrast, there was intense inflammatory cell infiltration whenever the external elastic lamina was penetrated with stent struts regardless of the stent type (Fig. 4, right panel).

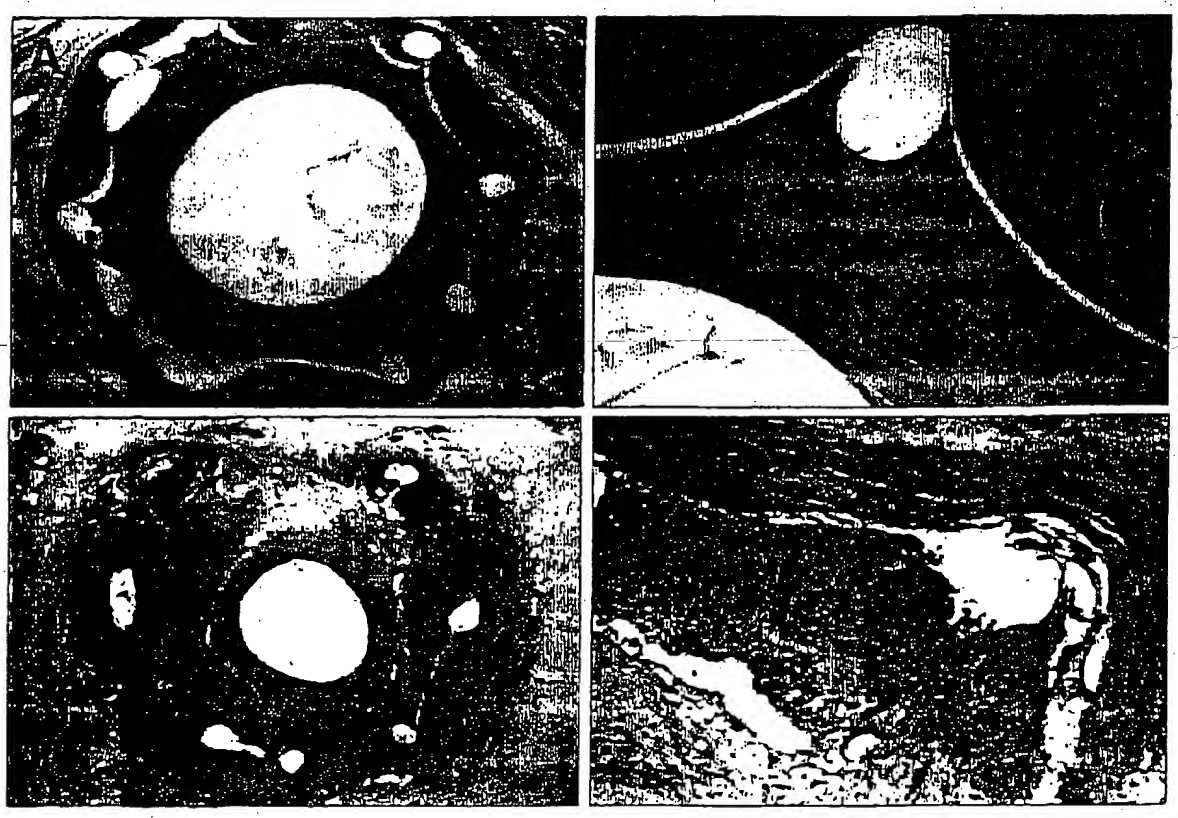

Fig. 4. Histopathologic findings of porcine coronary artery cross-section 28 days following control-bare stent or control-PU coating stent. 4-A (2.5X magnification) and 4-B (24X magnification) show no inflammatory responses in specimens in which stents were implanted without penetrating external elastic lamina. In contrast, 4-C (2.5X magnification) and 4-D (24X magnification) show intense inflammatory cell infiltration in specimens in which external elastic lamina was penetrated with stent struts. Hematoxylin and eosin stains.

There were no qualitative or quantitative differences in the inflammatory response to the 4 different stent types.

There were no significant differences in injury scores, luminal areas, intimal areas, medial areas, or intimal thicknesses among the 4 study groups (Table 1). Linear regression analysis of the relationship between neointimal thickness and injury score revealed no differences in the slope or intercepts of the regression lines for the 4 treatment groups (Table 2).

DISCUSSION

In the current study, we used sodium nitroprusside (SNP) as a nitric oxide donor, and impregnated it into a polyurethane (PU) polymer coated onto a metallic stent to evaluate the efficacy of sustained local delivery on reducing neointimal thickening in the porcine coronary artery stent overexpansion injury model. We demonstrated sustained biological effect of the eluted agent for up to 2 weeks in the porcine coronary artery, although neointimal hyperplasia was not reduced.

Besides the beneficial effects of metallic stents on recoil and adverse arterial remodeling, these devices could also serve as a drug-eluting reservoir. Several drugs such as heparin,[23-25] forskolin,[26] dexamethasone,[2] and taxol[27] have been assessed with stent coatings. A heparin-coated stent, in which heparin is covalently bonded to the stent and does not elute, may have reduced thrombogenicity, but did not reduce neointimal hyperplasia in a porcine coronary model.[24,25,28] Another polymer-coated stent, which delivered forskolin, was tested in the rabbit carotid injury model.[1,26] This device used a polyurethane polymer as a reservoir on a removable metallic stent and was able to deliver forskolin to the arterial wall in high local concentrations relative to the blood or other tissues. Although the delivered forskolin demonstrated vasodilating and antiplatelet biological activities, the tissue half-life was only 5.8 hours after removal of stent. In another

Table 1. Injury Scores and Morphometric Results in 4 Study Groups

Group	Control-bare (n=12)	Control-PU (n=11)	SNP-PU thin coating (n=12)	SNP-PU thick coating (n=11)
Injury score	2.14 ± 0.40	2.07 ± 0.32	2.36 ± 0.37	2.34 ± 0.36
Luminal area (mm^2)	0.48 ± 0.31	0.71 ± 0.38	0.44 ± 0.31	0.49 ± 0.34
Medial area (mm^2)	0.46 ± 0.20	0.46 ± 0.18	0.50 ± 0.23	0.57 ± 0.35
Intimal area (mm^2)	0.66 ± 0.20	0.68 ± 0.29	0.78 ± 0.23	0.85 ± 0.23
Intimal thickness (mm)	0.36 ± 0.14	0.35 ± 0.14	0.43 ± 0.12	0.46 ±0.12

n, number of vessels used for morphometric analysis; all measurements are expressed as mean ± standard deviation. SNP, sodium nitroprusside; PU, polyurethane; There were no significant differences among 4 study groups in injury scores, luminal areas, intimal areas, or intimal thicknesses.

Table 2. Linear Regression Analysis of Chronic Morphometric Measurements in Control-Bare Stents, Control-PU stents, and Nitric Oxide Eluting PU Stents on the Chronic Neointimal Thickening

	PU Control Stents		SNP-PU Thin Coating Stents		SNP-PU Thick Coating Stents	
	Coefficient	*p* value	Coefficient	*p* value	Coefficient	*p* value
Alpha (slope)	0.143	0.371	0.392	0.596	0.338	0.840
Beta (intercept)	-0.206	0.315	-0.326	0.629	-0.288	0.981

PU, polyurethane; SNP, sodium nitroprusside; Slope = 0.302; intercept = -0.290; R2 = 0.791.

study, a high molecular weight PLLA polymer was demonstrated to be well tolerated in porcine coronary arteries and was an effective means of providing sustained drug delivery for up to 4 weeks, although dexamethasone eluted from this stent did not reduce the neointimal hyperplasia in the porcine coronary injury model.[2] Several *in vitro* and *in vivo* studies of local paclitaxel delivery using polymer coating stent-based techniques to inhibit proliferation and lumen renarrowing have been performed with encouraging results.[27]

Although several synthetic polymers have been proposed to serve as vehicles for local drug delivery combined with metallic stents, a major issue raised by previous work using currently available biodegradable or non-biodegradable polymers is that of tissue-blood incompatibility.[29-31] Marked inflammatory cell responses were observed with several polymers implanted in porcine coronary arteries.[30] To be applicable to humans, long-term biocompatibility of a polymer would need to be assured. In the current study, no inflammatory response to the PU coated stents was observed relative to the bare stents. Deep penetration of stent struts to the adventitial layer, however, elicited marked inflammatory responses around the penetrating stent struts regardless of stent types, bare or polymer coated, while there was minimal or no inflammatory cell infiltration with lesser degrees of arterial injury.

Various agents that increase local nitric oxide production have been studied to restore normal endothelial function after arterial injury and reduce neointima formation.[10-14] Long-term dietary supplementation of L-arginine, the nitric oxide precursor, improved endothelium-dependent vaso-relaxation and reduced atherosclerotic lesion formation in rat[11] and hypercholesterolemic rabbit models.[10,12,13] Transfection of the endothelial nitric oxide synthetase gene in rats not only restored local nitric oxide production and vascular reactivity of the injured vessel, but also attenuated neointima formation.[14]

In this current study using a non-biodegradable PU coating, drug was uniformly dispersed within polymer forming a "monolithic matrix". Elution of nitroprusside by diffusion through pores of polymer was too rapid, occurring over only a few days.[26,32] Barrier coatings of two different thicknesses were therefore added to serve as a diffusion barrier, creating a polymeric "reservoir" to obtain controlled, sustained elution kinetics. By *in vitro* studies, these SNP-PU coating stents showed nearly zero order kinetics without an initial lag time; increased barrier thickness decreased the rate and prolonged the duration of elution. One design, the thick barrier coating, was used to prolong release of nitroprusside for the entire experimental period (4 weeks), while the thin barrier coating was used to maximize delivery to the first 2 weeks after injury, during which time the stimulus for proliferation would presumably be most intense.

The biological effect of the nitric oxide-eluting PU stents was demonstrated by measuring coronary arterial tissue cGMP levels. At time points of 1 day, 2 days, and 7 days after stent implantation, control (bare or PU) stented segments had lower cGMP levels than proximal or normal distal vessels, likely due to endothelial denudation, medial necrosis and cell loss at the sites of stent injury. SNP stented segments generally had higher levels than control stents, but cGMP levels were not normalized relative to proximal and distal normal segments at day 1 and 2. The failure of these stents to normalize cGMP levels at these time points may be related to a reduced target cell mass due to endothelial and medial injury, loss of some drug from polymer surface facing arterial lumen and thus not apposed to vessel wall, or a uniform elution rate with no early "burst" of nitric oxide release. By day 7, however, cGMP levels were normalized relative to proximal and distal normal artery in SNP stented segments, but remained low in control stented segments. This finding suggests regrowth of medial and intimal tissue by this time point, providing target cells for nitric oxide and encapsulating the stent wires, "capturing" most of the nitric oxide released from the polymer matrix. Despite probable reconstitution of medial and intimal tissue layers, these layers did not appear to be functionally normal by 7 days, as evidenced by continued suppression of cGMP levels in control stented segments compared with proximal and distal normals. By day 14, control stented segments showed restoration of cGMP levels relative to proximal or distal

normals. The greater cGMP levels in control stented segments may be due to tissue healing response and neointimal hyperplasia. SNP stented segments showed supranormal levels of cGMP, which were significantly higher than control stented segments, suggesting continued release of nitric oxide from the stents and its biological action on hyperplastic tissue. At all time points, proximal and distal normal segments did not show increased cGMP levels in arteries treated with SNP stents compared with controls, confirming the local nature of delivery of nitric oxide to the stented arterial wall by the SNP stents.

Neither SNP stent design reduced neointimal hyperplasia, despite prolonged biological activity demonstrated through elevation in tissue cGMP levels. Potential explanations include an inadequate dose of nitric oxide delivered to the tissue, particularly during the first few days, when cGMP levels were increased but not normalized. Modification of the stent design, with SNP dispersed within the barrier coating to provide an early "burst", might improve efficacy in this regard. The degree of arterial injury may have been too great in some vessel segments with inflammatory response due to penetration of the EEL in many segments which may have "overwhelmed" the effect of nitric oxide.[13] Finally, the hypothesis that nitric oxide is an important modulator of arterial response to injury, at least in this species and model, may be incorrect.

In conclusion, stent-based local delivery of sodium nitroprusside from an eluting stent was effective in delivering nitric oxide in sustained fashion for up to 4 weeks *in vitro* experiments and demonstrated biologic effectiveness through increased local tissue cGMP levels for up to 14 days after stent implantation. However, this sodium nitroprusside-eluting stent failed to reduce chronic neointima thickening in the porcine coronary stent injury model. This technology may prove more efficacious, however, with improved drug elution characteristics or another choice of eluted drug.

ACKNOWLEDGMENTS

The authors wish to acknowledge the Dr. Edward Plow and Mr. Timothy Burke for their generous technical assistance and use of laboratory facilities.

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EXHIBIT C

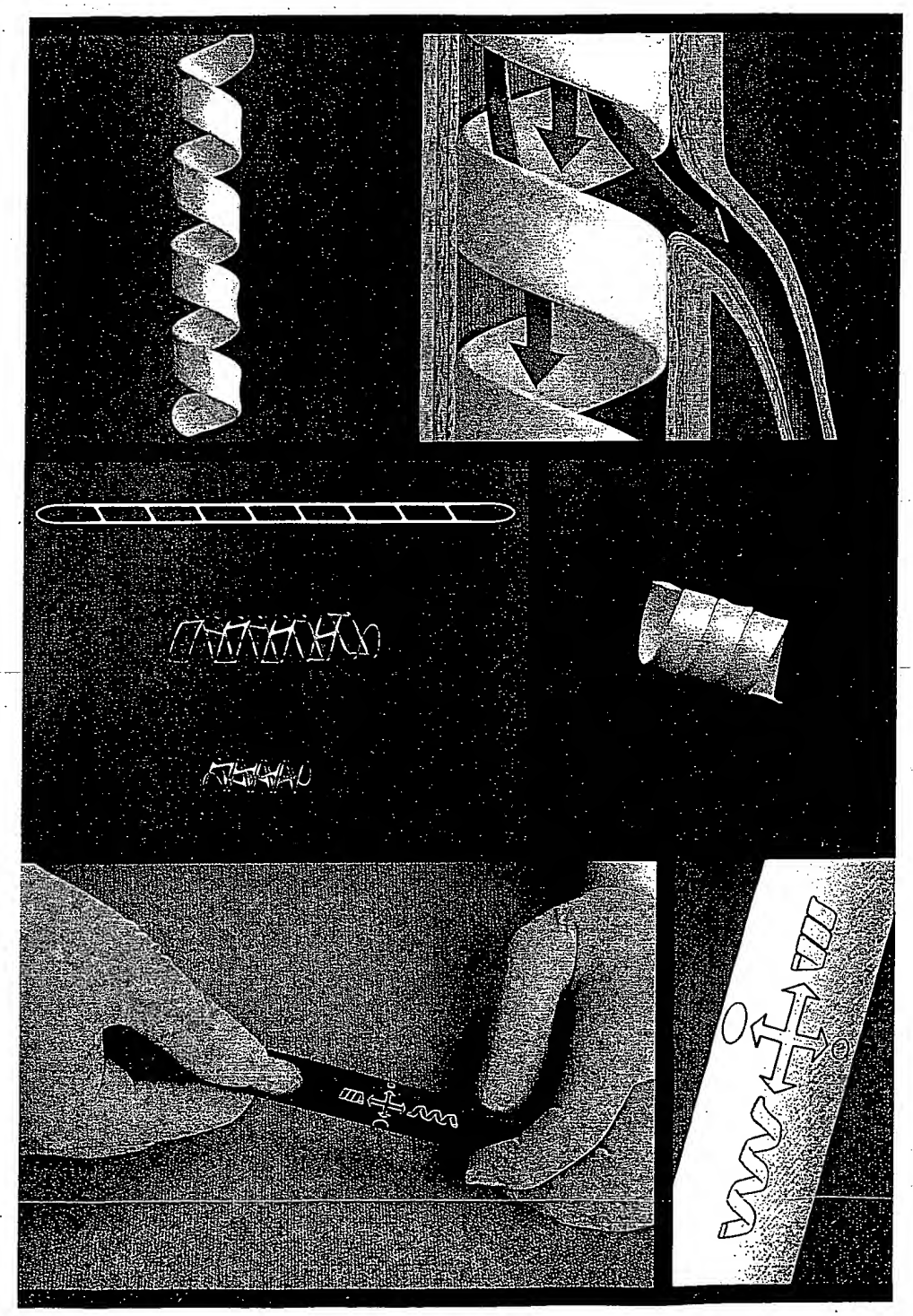
aSpire™ Covered Stent

VASCULAR ARCHITECTS®

Preserving Native Structures

PRESERVES NATIVE STRUCTURES

The unique design of the aSpire Covered Stent allows the physician to provide exceptional coverage while simultaneously protecting side branches of the lumen.

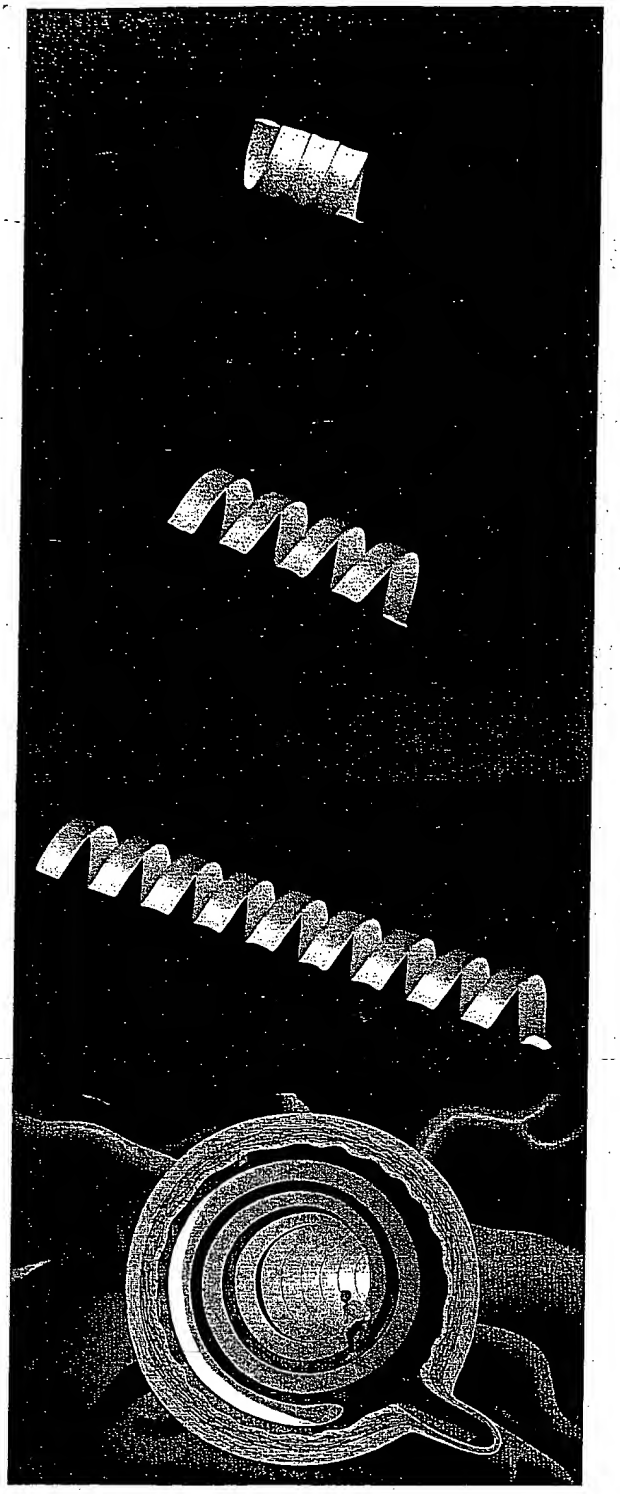


NO COMPROMISE DESIGN

The revolutionary design of the aSpire Covered Stent provides exceptional radial strength, flexibility and conformability. The crush resistant nitinol frame is fully covered by ePTFE delivering significantly enhanced luminal coverage.

CONTROLLED EXPANSION™ DELIVERY SYSTEM

The aSpire Covered Stent offers a unique delivery system that provides precise placement of the stent. The Controlled Expansion feature allows physicians to accurately position the stent, fully appose it to the lumen wall and reposition if necessary prior to deployment.



aSpire Covered Stent

Catalog Number	Diameter	Length
9C-06-030	6 mm	3.0 cm
9C-07-025	7 mm	2.5 cm
9C-08-025	8 mm	2.5 cm
9C-09-025	9 mm	2.5 cm
10C-10-025	10 mm	2.5 cm
10C-11-025	11 mm	2.5 cm
10C-12-025	12 mm	2.5 cm
9O-06-050	6 mm	5.0 cm
9O-07-050	7 mm	5.0 cm
9O-08-050	8 mm	5.0 cm
9O-09-050	9 mm	5.0 cm
10O-10-050	10 mm	5.0 cm
10O-11-050	11 mm	5.0 cm
10O-12-050	12 mm	5.0 cm
11O-13-050	13 mm	5.0 cm
11O-14-050	14 mm	5.0 cm
9O-06-100	6 mm	10.0 cm
9O-07-100	7 mm	10.0 cm
9O-08-100	8 mm	10.0 cm
10O-09-100	9 mm	10.0 cm
11O-10-100	10 mm	10.0 cm

Preserving Native Structures.

Vascular Architects is reshaping the treatment of obstructive disease with interventional devices built to preserve native structures. For more information on the aSpire or Vascular Architects' product line, please contact us toll-free at 877-827-2728, or email us at customers@vascularchitects.com

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Caution: Federal law restricts this device to sale by or on the order of a physician.

ML 60055 Rev. A

Exhibit D

Attorney Docket No.: **VASC 1020-2 US**

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application Inventor: Bruce J Barclay, et al. SC/Serial No.: **09/910,703** Confirm. No.: 2083 Filed: 20 July 2001 Title: Biologically Active Agent Delivery Apparatus and Method	Group Art Unit: **3738** Examiner: **Pellegrino, Brian B.** Customer No. 22470

DECLARATION OF KIRTI P. KAMDAR

Mail Stop AF
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Kirti P. Kamdar, one of the joint inventors for this application, declare as follows.

I am Vice President, R&D for the assignee of this application, Vascular Architects, Inc. I was responsible for having an experimental study conducted to determine the extent and duration of nitric oxide (NO) release of the aSpire® covered stent containing a NO generator under dynamic flow conditions and am familiar with the procedures followed and the results obtained out this study. Twelve aSpire® covered stents (stent grafts) were divided into three groups. Group 1 consisted of 5 aSpire® covered stents containing a first NO-eluting mixture. Group 2 consisted of 5 aSpire® covered stents containing a second NO-eluting mixture. Group 3 consisted of 2 control aSpire® covered stents. The two NO-eluting stent groups consisted of two different formulations of sodium nitroprusside (SNP), a NO donor, within a liquid silicone carrier. The testing proceeded as follows:

stent groups consisted of two different formulations of sodium nitroprusside (SNP), a NO donor, within a liquid silicone carrier. The testing proceeded as follows:

1. ePTFE tubing was placed onto 12 Nitinol stent frames;
2. the appropriate NO-eluting mixture was injected inside the ePTFE tubing for each of Groups 1 and 2; Group 3 containing no SNP or silicone;
3. the ends of ePTFE tubing were sealed providing 10 aSpire® covered stents (Groups 1 and 2) with NO-eluting mixtures sandwiched between two layers of ePTFE and surrounding the Nitinol stent frame;
4. each of the 12 aSpire® covered stents (12 mm x 5 cm) was placed into a separate testing chamber, each testing chamber connected to a separate circulatory system;
5. each testing chamber was then connected to a separate liquid reservoir containing phosphate buffered solution (PBS) to create a closed-loop circulating (100 ml/min.) test system;
6. each testing chamber, containing one of the 12 aSpire® covered stents, was then filled with the test liquid;
7. the test liquid was then circulated through each reservoir to permit the NO-eluting mixture to pass NO through ePTFE and into the test liquid of each testing chamber;
8. samples were taken periodically for 67 days to measure the amount of NO being released into each testing chamber; and
9. plots of NO vs. time were generated for Elution Data (the results of Groups 1 and 2 plotted separately) and T 1/2 (single plots for Group 1 and 2 plus a best-fit curve), attached as Exhibits E and F.

The results show that NO can be delivered for extended periods of time (more than 60 days) in physiologically effective amounts for the 12mm x 5cm aSpire® covered stent containing an NO-eluting mixture. The procedure for doing so is relatively straightforward

and can be tailored to provide an improved or optimal delivery rate by, for example, adjusting the amount and concentration of the NO generator within the NO-eluting mixture.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States Code, § 1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Kirti P. Kamdar

Date: 25 July 2003

HAYNES BEFFEL & WOLFELD LLP
P.O. Box 366
Half Moon Bay, CA 94019
Telephone: 650-712-0340/Facsimile: 650-712-0263

EXHIBIT E

VASCULAR ARCHITECTS

Elution Data – Dynamic Model

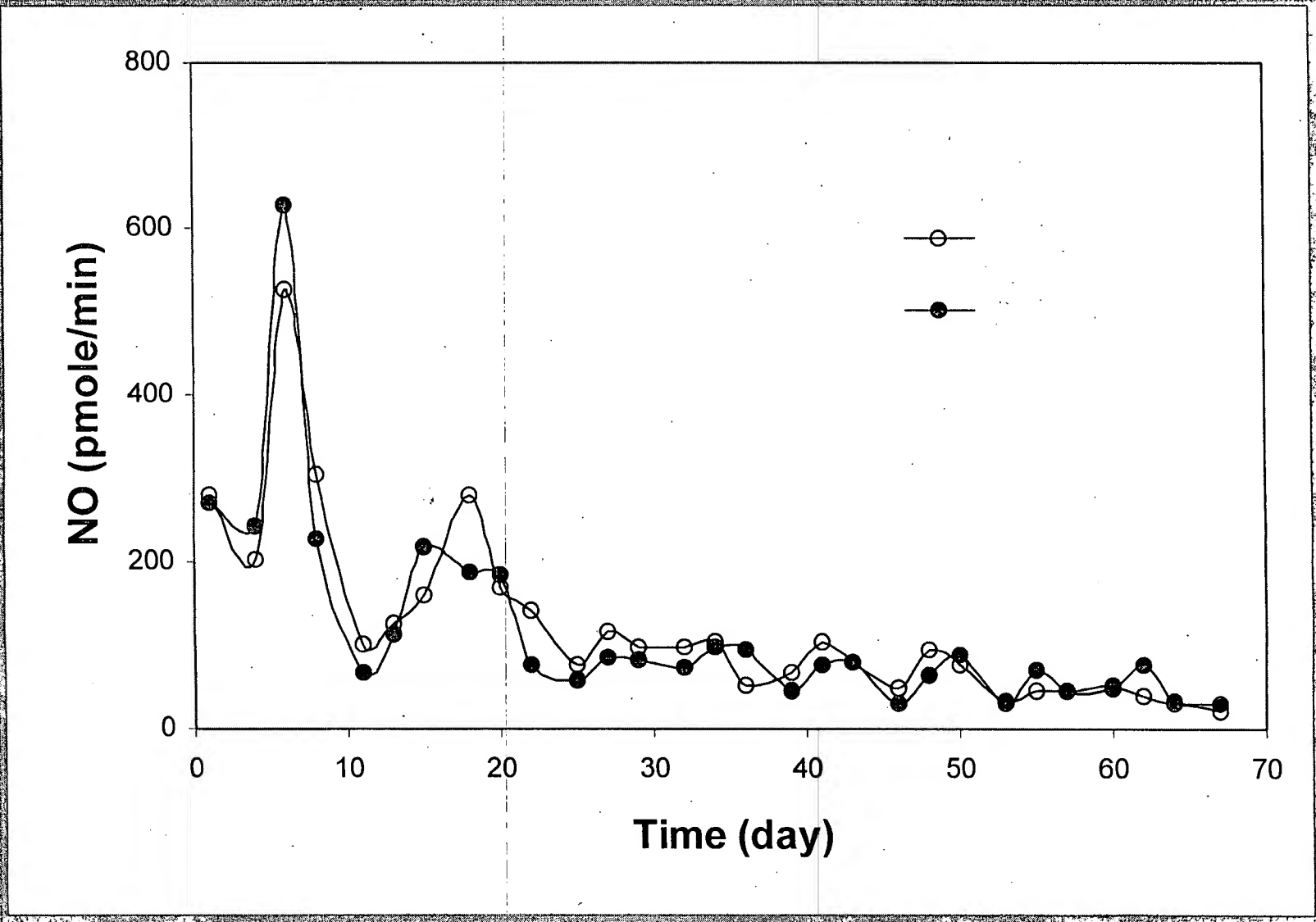

EXHIBIT F

VASCULAR ARCHITECTS

T ½ - Dynamic Model